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Identification of COL4A5 defects in Alport's syndrome by immunohistochemistry of skin

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Background. The COL4A3-COL4A4-COL4A5 network in the glomerular basement membrane is affected in the inherited renal disorder Alport's syndrome (AS). Approximately 85% of the AS patients are expected to carry a mutation in the X-chromosomal COL4A5 gene and 15% in the autosomal COL4A3 and COL4A4 genes. The COL4A5 chain is also present in the epidermal basement membrane (EBM). It is predicted that approximately 70% of the COL4A5 mutations prevent incorporation of this chain in basement membranes.

Methods. We investigated whether or not COL4A5 defects could be detected by immunohistochemical analysis of the EBM. Punch skin biopsies were obtained from 22 patients out of 17 families and two biopsy specimens from healthy males were used as controls.

Results. In four cases with the COL4A5 frameshift or missense mutations, the COL4A5 chain was either lacking from the EBM (male) or showed a focally negative pattern (female). In three other patients with a COL4A5 missense mutation, a COL4A3 and a COL4A4 mutation, respectively, the COL4A5 staining was normal. A (focally) negative EBM-COL4A5 staining was found in three patients of six families with a diagnosis of AS and in one family of a group of four families with possible AS.

Conclusions. The (focal) absence of COL4A5 in the EBM of skin biopsy specimens can be used for fast identification of COL4A5 defects. Combined with polymorphic COL4A5 markers, both postnatal and prenatal DNA diagnosis are possible in the family of the patient.

Type IV collagen, a multimeric protein composed of three α chains, forms a macromolecular network, which is the main structural component of the glomerular basement membrane (GBM). These type IV collagen chains consist of a central collagenous domain of approximately 1400 amino acid residues and noncollagenous (NC) domains at the N- and C-terminal ends, called the 7S and the NC domain, respectively. A total of six type IV collagen chains, COL4A1 through COL4A6, with molecular weights ranging from 170 to 185 kDa, has been identified [1–4]. Three α(IV)-chains form a triple helix (trimer), which starts at the C-terminal NC domain. The N-terminal 7S domains and the NC domains of different triple helices associate to form a "chicken-wire" shaped meshwork. In the GBM, two collagen type IV networks have been identified: the first consists of COL4A1 and COL4A2 trimers, whereas the second network comprises COL4A3, COL4A4, and COL4A5 chains [5, 6]. The COL4A6 chain is not present in the GBM [7]. Heterotrimers and homotrimers of COL4A1 and COL4A2 are ubiquitously present in all basement membranes [6], whereas COL4A3, COL4A4, COL4A5, and COL4A6 have a restricted tissue distribution [8–12]. For example, in skin, only COL4A1, COL4A2, COL4A5, and COL4A6 are found in the epidermal basement membrane (EBM) [7, 11].

Alport's syndrome (AS) [13] is a clinically heterogenous, inherited disorder of the GBM that is characterized by hematuria, proteinuria, progressive renal failure, and high-tone sensorineural hearing loss [14–17]. Other clinical features such as ocular abnormalities (lenticonus and retinal anomalies), diffuse esophagal or vulvar leiomyomatosis [18, 19], and macrothrombocytopenia [20, 21] have also been described. Electron microscopic analysis of renal biopsy specimens of AS patients shows an irregular thinning/thickening and multilamellation of the GBM. In young patients, thinning of the GBM can be the only symptom [22, 23]. The disorder is genetically heterogenous, but a vast majority (85%) of Alport pedigrees showed X-linked dominant inheritance (XL-AS) and was caused by mutations in the COL4A5 gene located in the Xq22-24 region [15, 24–26]. The estimated gene frequency of AS is 1:5000 [27, 28]. Approximately 15%

[1] **See Editorial, p. 1575.**

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of the pedigrees showed an autosomal recessive pattern of inheritance of AS (AR-AS), caused by mutations in the COL4A3 and COL4A4 genes at chromosome 2q35-37 [29, 30]. Autosomal dominant forms of AS have been mapped to the COL4A3 and COL4A4 locus, but mutations have not yet been identified [31].

The heterogenous nature of AS and the huge size of the type IV collagen genes involved hamper the mutation analysis. As the COL4A5 protein is present both in the GBM and in the EBM, we tested whether immunohistochemical (IHC) analysis of skin biopsy specimens with antibodies against the COL4A5 chain could be used to identify a COL4A5 gene defect. Approximately 70% of the reported mutations in the COL4A5 gene are predicted to lead to a truncated protein with an absent or nonfunctional NC domain, which prevents triple-helix formation and incorporation in the BM network. We investigated patients with established type IV collagen mutations, patients with the clinical diagnosis of AS, and patients with possible AS or familial benign hematuria (FBH).

METHODS

Families/patients

Clinical criteria for AS were as described before [16]. DNA was extracted by a simple salting out procedure from peripheral blood lymphocytes of patients and family members [32].

Mutation analysis and polymorphic markers

Individual exons of the COL4A5 gene of patients with AS were screened for mutations by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis [33], using the Genephor system (Amersham Pharmacia Biotech, Uppsala, Sweden). In case of mobility shift, direct sequence analysis of the exon was performed using the Taq Dyedeoxy™ Terminator protocol from the manufacturer and the ABI 377 Automated Sequencer (Applied Biosystems, Foster City, CA, USA). Polymorphic microsatellite markers (DXS178; COL4A5-2B6) were used as described before [30].

Indirect immunofluorescence analysis and panel of antibodies

Punch skin biopsy specimens were obtained from 22 patients out of 17 families by a standard procedure, immediately frozen in isopentane (−80°C), and stored at −80°C or in liquid nitrogen. Two skin biopsy specimens of healthy males were used as control specimens. The specimens were embedded in OCT™ (10.24% polyvinyl alcohol, 4.26% polyethylene glycol, 85.5% nonreactive ingredients; Tissue Tec, Miles, Naperville, USA). Five μm cryosections were taken up on glass slides (Superfrost plus; Menzell Gläser, Braunschweig, Germany) that subsequently were air dried at room temperature for one night before use. The monoclonal antibodies used in this study required different treatment of the sections, as indicated in Table 1. The sections were washed with phosphate buffered saline (PBS; 137 mM NaCl, 13 mM $Na_2HPO_4 \cdot 2H_2O$, 3 mM KH_2PO_4 in demineralized water, pH 7.4) before the primary antibodies were applied. After one hour of incubation in a humidified chamber (room temperature), the sections were washed three times with PBS (5 min each), and a secondary, fluorescein isothiocyanate- or Texas Red-conjugated goat-anti-mouse antibody (Southern Biotechnology Associates Inc. Birmingham, AL, USA) was applied. Sections were incubated for 45 minutes at room temperature, washed three times (for 5 min each) with PBS, mounted in Mowiol (Hoechst, Frankfurt, Germany), and examined using a Zeiss microscope equipped with epifluorescence illumination optics. Skin biopsy specimens from normal individuals were included as the controls for each procedure.

The panel of monoclonal antibodies used in this study (Table 1) can be used for the IHC analysis of both skin biopsy and kidney biopsy specimens. In the case of IHC of skin biopsy specimens, the panel includes two different procedures for COL4A5 staining (MAB5 on urea/glycine treated sections and Moab A7 for Tris/urea treated sections), one positive "control" staining for COL4A1 (MAB1), which indicates both the localization of the EBM and the quality of the section, two negative control stainings with primary antibodies against COL4A3 (MAB3, MCA-p1), and one control staining in which the primary antibody is replaced by PBS.

RESULTS

Staining the epidermal basement membrane of patients

Sections of controls and patients were positive for COL4A1 (Fig. 1d, g), with an uninterrupted staining pattern of the EBM, and negative for COL4A3 (Fig. 1e, h) and the PBS control (Fig. 1f). The EBMs in these control sections were also positive for COL4A5 (Fig. 1i). Identical results were obtained with the MAB5 and the Moab A7 procedure. A male patient (A; Table 2) with a frameshift mutation in exon 49 (5005 + 1Gins10) was completely negative for COL4A5 (Fig. 1a), whereas a female patient (B) with a frameshift mutation (3723delA; Table 2) in exon 39 showed a mosaic staining pattern (Fig. 1b). The X-inactivation pattern resulted in focally negative and positive parts of the EBM. Two male patients (C, D) with missense mutations resulting in glycine substitutions (C: exon 31, 2808G > A, Gly869Glu; D: exon 23,1782G > A, Glu527Gly) showed a negative staining pattern, whereas a male patient (E) with a missense mutation (exon 7, 588G > A, Gly129Glu) showed a normal staining pattern (Fig. 1c). The EBM of a female

Table 1. Monoclonal antibodies, section pretreatment and references

Antigen	Antibody	Dilution	Section treatment	Reference
COL4A1	MAB1	1:10–25	10 min acetone −20°C	[12, 38, 39]
COL4A3	MAB3	1:10–25	10 min acetone −20°C	[20, 38, 39]
COL4A3	MCA-pl	u.d.–1:5	15 min cold Tris/urea	[40]
COL4A5	MAB5	1:10–25	10 min acetone −20°C + 5 min cold glycine/urea	[12, 38, 39]
COL4A5	Moab A7	u.d.–1:5	15 min cold Tris/urea	[41]

Abbreviation u.d. is undiluted culture supernatant.

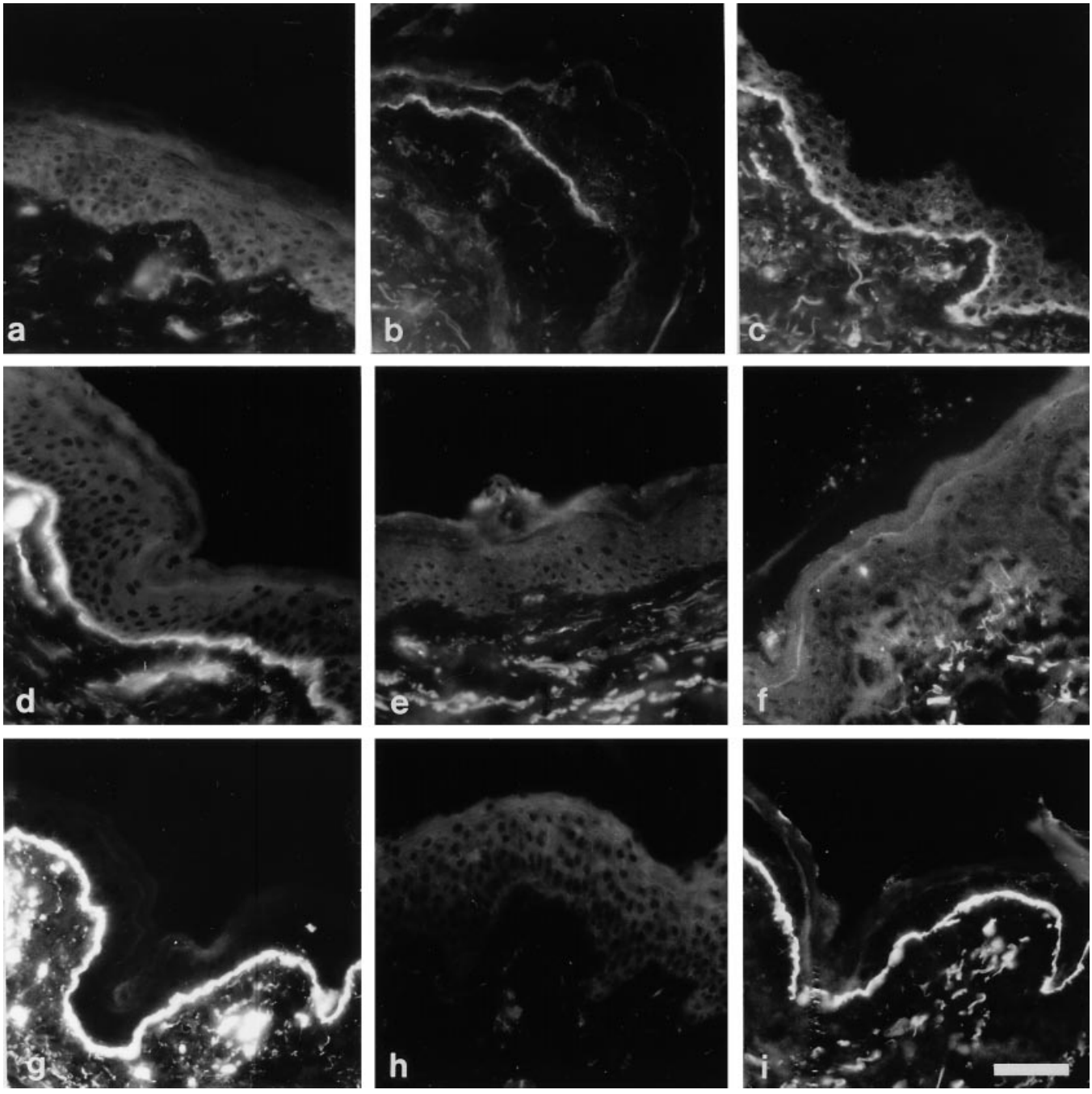

Fig. 1. Immunohistochemistry of skin biopsies of AS patients (*a–e*) and normal skin (*f–i*). Sections were stained with monoclonal antibodies against COL4A5 (a, b, c, i), COL4A1 (d, g), COL4A3 (e, h) or were incubated with PBS instead of a monoclonal antibody (f; negative control). Bar indicates 50 μm.

Table 2. Immunohistochemistry of the epidermal basement membrane (EBM) in patients with Alport's syndrome (AS) and possible AS

Family	*N*	M/F	Clinical diagnosis	COL4A5 staining	Position mutation *gene/exon*	Nature of mutation	Effect on coding sequence	Reference
I. Patients with established mutations								
A	1	M	AS	negative	COL4A5/49	5005 + 1Gins10	splice site	[27]
B	1	F	AS	mosiac	COL4A5/39	3723delA	frame shift	this study
C	1	M	AS	negative	COL4A5/31	2808G>A	Gly869Glu	this study
D	1	M	AS	negative	COL4A5/23	1782G>A	Gly527Glu	this study
D	1	F	AS	mosiac				
E	1	M	AS	positive	COL4A5/07	588G>A	Gly129Glu	[34, 35]
F	1	F	AS	positive	COL4A3/05 (homozygote)	4414del5/ 4419del5	frame shift & stop 33 codons downstream	[36]
G	1	M	FBH	positive	COL4A4	2898G>A	Gly897Glu	[37]
II. Patients diagnosed AS								
H	2	M	AS	positive	—	—	—	—
I	1	M	AS	positive	—	—	—	—
J	1	M	AS	negative	—	—	—	—
K	1	M	AS	negative	—	—	—	—
L	1	F	AS	positive	—	—	—	—
III. Possible AS & FBH patients								
M	2	M	possible AS	negative	—	—	—	—
N	1	M	possible AS	positive	—	—	—	—
O	1	M	possible AS	positive	—	—	—	—
P	1	M	possible AS	positive	—	—	—	—
Q	1	M	FBH	positive	—	—	—	—

carrier (D) showed a mosaic staining pattern. These data, summarized in Table 2 part I, indicate that IHC of the EBM for COL4A5 is suitable to identify COL4A5 mutations that prevent incorporation of the COL4A5 chain in type IV collagen BMs. The patients with mutations in COL4A3 (F) and COL4A4 (G) showed a normal COL4A5 staining pattern in the EBM.

Sections of skin biopsy specimens of six patients with the diagnosis AS were stained for COL4A5 (H–L; Table 2, part II). The diagnosis was based on the presence of at least three of the four clinical criteria for AS [16]. The familial cases were likely X linked, based on the presence of less severely affected female patients, exclusion of the COL4A3/COL4A4 locus, and if the family was large enough, on linkage analysis for the COL4A5 locus. The sections of patients in two families (J, K; $N = 2$) were negative for the COL4A5 chain. Patients in three other (H, I, L; $N = 4$) had a normal COL4A5 staining pattern in the EBM. Skin biopsy specimens of five persons from four families (M–P) with an ambiguous diagnosis of AS were also investigated. One patient (M) showed a negative staining for COL4A5. This result was confirmed in a second patient in this family. The other three patients (N–P) showed a normal COL4A5 staining pattern. The skin biopsy specimen of a FBH patient (Q) was also normal, and COL4A5 was present in the EBM (Table 2, parts I and III).

Cumulative results of all families with an evident diagnosis AS indicate that in 6 (A–D, J, K) out of 11 unrelated Alport patients (A–F and H–L), a COL4A5 defect was detectable by a negative or mosaic COL4A5 staining pattern of the EBM. No false diagnosis was obtained in the AR-AS and FBH cases (F, G, Q), and the COL4A5 staining of the EBM was normal in all three patients. The results obtained in the group of possible AS families (M–P) did not contribute to the overall detection rate because the diagnosis of AS was not clinically certain.

Family investigations

Immunohistochemical analysis of skin biopsies of male patients in combination with marker analysis can be used to determine the COL4A5 risk haplotype in a family without the necessity of performing extensive linkage analysis. In case of a female patient, a second patient must to be available to determine the phase of the risk haplotype. Subsequently, postnatal and prenatal investigations can be offered in such a family. If a *de novo* mutation is suspected, then additional skin biopsies have to be performed in family members who are at risk. Recently, we used this approach in two families (D and J; Fig. 2). DNA and skin biopsies were obtained in family D from a carrier (*II:2*) and her severely affected brother (*II:1*). The carrier turned out be informative for marker DXS178, and a prenatal diagnosis could be offered on the basis of this marker. In the second family (J), a severely affected male patient (*II:1*) was tested with his asymptomatic sister (*II:2*) and mother (*I:2*). The EBM of the patient was negative for COL4A5, whereas the EBM of both women was positive. The sister did not inherit the risk haplotype. The IHC indicated that most likely a *de novo* COL4A5 mutation had occurred in the male patient. Skewed X inactivation in the mother

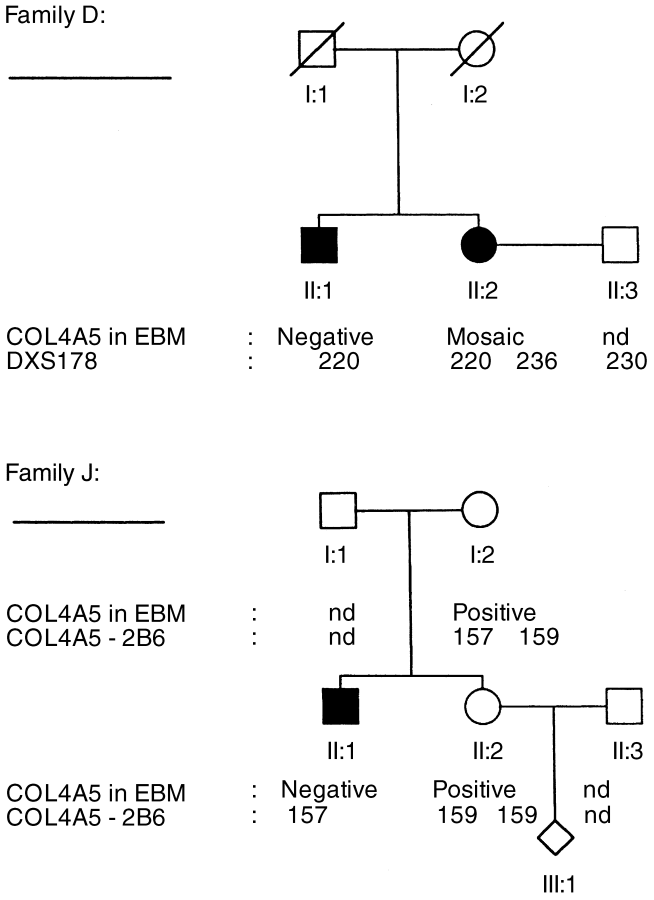

Fig. 2. Family investigations using immunohistochemistry of skin biopsy specimens and polymorphic COL4A5 markers: pedigrees of families D and J. Symbols are: (□) unaffected male; (■) affected male; (○) unaffected female; (●) affected female; (/) deceased; (◇) fetus. The abbreviation nd means not determined.

cannot be entirely excluded, although we did not observed this in our series of female patients.

DISCUSSION

Mutations in the X-chromosomal gene coding for COL4A5, a component of the heterogenic type IV collagen network of the GBM, are expected to be involved in approximately 85% of all cases of AS. Thus far, no other renal disease with a mutation in the COL4A5 gene has been reported, and no other X-linked gene has been found solely mutated in AS. A large part of the COL4A5 mutations is predicted to prevent integration into type IV collagen containing BMs, such as the GBM and the EBM [12, 42]. IHC analysis of skin biopsy specimens as a rapid method for the diagnosis of XL-AS has been described before, but so far no studies on skin biopsy specimens of patients with characterized mutations have been published [7, 10, 41, 43–46]. It is estimated the underlying COL4A5 mutation can be detected by IHC of the skin that in at least 50 to 60% of the AS patients [42]. A skin biopsy is less invasive and easier to perform than a kidney biopsy. Therefore, combining the criteria for AS, as Flinter et al defined [16], with IHC of the skin can be the first step in the diagnostic protocol of AS.

First, we tested the EBM of patients with characterized mutations, either in the COL4A5, COL4A3, or COL4A4 gene. The epitopes of the monoclonal antibodies used were located in the NC domain at the carboxy-terminal part of the type IV collagen chains. The patients with a COL4A5 frameshift mutation showed a negative (male; A) or mosaic negative (female; B) pattern, indicating that the resulting truncated chain could not be incorporated (or detected) in the COL4A5-COL4A6 network. It has been reported that in case the α5(IV) chain is absent from the EBM, the α6(IV) chain is also absent [7, 44, 45] and the entire COL4A5-COL4A6 network in these patients might be (focally) absent. In the male patient, the mutation (COL4A5/exon 49) occurred in the NC domain itself. Therefore, the negative staining of the EBM in this patient could be due to the absence of the entire network or to the absence (or the alteration) of the epitopes in the NC domain. In this case, as well as in similar cases with mutations in the COL4A5 NC domain, it would be appropriate to test the EBM with monoclonal antibodies against COL4A6 to check whether there is a COL4A5-COL4A6 network present. We have hypothesized for this patient (A) that the remaining part of the COL4A5 NC domain could be sufficient for integration of this α chain in the COL4A3-COL4A4-COL4A5 network, which would explain the relatively mild course of the disease [34, 35].

The EBM of three male patients with COL4A5 missense mutations (C–E) showed either a negative (C, D) or a positive (E) staining pattern. The mutations in exon 31 (C) and exon 23 (D), resulting in glycine into glutamic acid substitutions, are located in the collagenous domain of COL4A5 and interrupt the characteristic Gly-X-Y repeat. Why these mutations lead to the absence of the COL4A5-COL4A6 network and not just result in an altered triple helix is not clear. Further investigations are necessary to establish the exact effect of these mutations and will probably allow further discrimination in the group of predicted glycine substitutions. The patient (E) with the missense mutation in COL4A5/exon 7 was COL4A5 positive, indicating that this amino acid substitution did not prevent incorporation in the COL4A5-COL4A6 network and that the epitopes of the two monoclonal antibodies to COL4A5 were present. As expected [39], the two patients with established mutations in COL4A3 (F) and COL4A4 (G) showed a normal positive COL4A5 staining in the EBM.

Skin biopsy specimens of patients from two families with AS (J, K) showed a negative or mosaic staining pattern, indicating the presence of a COL4A5 mutation.

A positive COL4A5 staining in the other three families (H, I, L) does not exclude a COL4A5 defect. Definite proof for the involvement of COL4A5 has to come in these cases from mutation analysis. From our group of four families with possible AS (M–P), one family (M) was identified as having a COL4A5 mutation.

Immunohistochemical analysis of the EBM can identify COL4A5 defects in approximately 50 to 60% of the patients with Alport's syndrome [42], which is compatible with our results, although only a small group of patients has been tested. The results of our study were compared with the cumulative results of seven previous studies [7, 10, 41, 43–46]. In these studies, involving 30 AS families, 32 of 36 male patients and 32 of 34 female patients were detected by IHC. All 25 XL-AS families and three out of five *de novo* AS families [43] were identified. In 12 of these families, nonaffected members were tested, and all EBMs were COL4A5 positive. The detection rate in the AS families in our study (6 out of 11 families) is lower than the overall detection rate of these seven studies. Because our study almost exclusively included the probands of the families, and the other studies (*a*) did not include data of XL-AS families that were not detected by IHC and (*b*) included more than one patient per family, there is a bias in the results from the literature. Gubler et al reported COL4A5-positive EBMs in patients from four AR-AS families [39]. The EBM of the proband of our AR-AS family (F) is also COL4A5 positive. Nakanishi et al described IHC of the EBM in a group of four possible AS families, but COL4A5-negative EBMs were not detected [43]. The data show that the detection of both male patients and female carriers by skin biopsy specimen analysis is a fast and reliable method to diagnose COL4A5 defects causing XL-AS. The score of IHC analysis of the EBM, an *in vivo* protein truncation test, is comparable with the score of mutation detection by PCR-SSCP [27]. Mutation detection of COL4A5 is slow, laborious, and expensive because all 51 exons have to be screened individually, whereas skin biopsy analysis is fast, less expensive, and less laborious. Recently, Nakanishi et al studied the correlation between the severity of the disease and the degree (or the ratio) of α5 chain expression in the EBM of 25 female patients from 17 families [47]. This study indicates that IHC examination of the EBM may also be a useful method for predicting the disease severity in female patients.

The detection rate of COL4A5 mutations by IHC analysis of skin biopsies could become higher if monoclonal antibodies directed against epitopes in the 7S domain or the collagenous domain of COL4A5 were available. In such a case, the antibodies would not be used to study the absence of the chain, but the absence of an epitope, because of a mutation. Such monoclonal antibodies could also indicate the position of COL4A5 mutations, making mutation analysis less laborious. However, because of the repetitive and conserved amino acid sequence of the collagen type IV chains, it will be very difficult to generate chain specific monoclonal antibodies to these regions. Antisera against COL4A6, the second component of the COL4A5-COL4A6 network in the EBM, could indicate whether the absence of a COL4A5 signal in the EBM is caused by the complete absence of this network (both COL4A5 and COL4A6 negative) [7] or by the absence of the epitope in the COL4A5 NC domain (COL4A5 negative and COL4A6 positive). This may have implications for clinical manifestations of the genetic defect. Apart from AS with leiomyomatosis, thus far no other renal diseases have been reported that are linked with COL4A6 mutations.

A mutation in COL4A5 can have different effects in the COL4A3-COL4A4-COL4A5 network of the GBM compared with the COL4A5-COL4A6 network of the EBM [46]. This is also a possible explanation for not detecting certain COL4A5 mutations by immunostaining of the skin. The presence of COL4A5 in the EBM in combination with the absence of this chain in the GBM can also be the result of mutations in the COL4A3 or COL4A4 genes in autosomal AS. The absence of COL4A5 in the EBM is a clear indication for XL-AS, but the presence of COL4A5 does not exclude XL-AS, nor does it unequivocally determine the patient to have AR-AS.

In summary, the combination of clinical data and immunostaining of skin biopsies can identify a large portion of AS patients, saving these patients from undergoing a kidney biopsy. This approach is fast and does not require the clinical data and/or cooperation of several family members (as is the case with linkage analysis), and the percentage of mutations detected is at least equal to the percentage of mutation detection by PCR-SSCP analysis. Because IHC localizes the gene defect to COL4A5, it can be used in combination with COL4A5-linked DNA markers to enable postnatal and prenatal diagnosis in suitable families and to identify *de novo* mutations.

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APPENDIX

Abbreviations used in this article are: AR-AS, autosomal recessive pattern of inheritance of AS; AS, Alport's syndrome; EBM, epidermal basement membrane; FBH, familial benign hematuria; GBM, glomerular basement membrane; IHC, immunohistochemical; NC, noncollagenous; PBS, phosphate buffered saline; XL-AS, X-linked dominant inheritance.

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